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P-body components LSM1, GW182, DDX3, DDX6 and XRN1 are recruited to WNV replication sites and positively regulate viral replication

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ABSTRACT

In mammalian cells, proteins involved in mRNA silencing and degradation localize to discrete cytoplasmic foci called processing or P-bodies. Here we show that microscopically visible P-bodies are greatly diminished following West Nile viral infection, but the component proteins are not depleted. On the other hand, many P-body components including LSM1, GW182, DDX3, DDX6 and XRN1, but not others like DCP1a and EDC4 are recruited to the viral replication sites, as evidenced by their colocalization at perinuclear region with viral NS3. Kinetic studies suggest that the component proteins are first released from P-bodies in response to WNV infection within 12 h post-infection, followed by recruitment to the viral replication sites by 24–36 h post-infection. Silencing of the recruited proteins individually with siRNA interfered with viral replication to varying extents suggesting that the recruited proteins are required for efficient viral replication. Thus, the P-body proteins might provide novel drug targets for inhibiting viral infection.

Introduction

West Nile virus (WNV) is a mosquito borne flavivirus that can cause serious and potentially fatal infection of the central nervous system, with up to 10% mortality in humans (Mackenzie et al., 2004; Rappole et al., 2000). Currently there is neither a specific treatment nor an approved vaccine for this infection. The viral genome is a single stranded positive sense RNA of ~11 kb that encodes a single open reading frame flanked by 5′ and 3′ untranslated regions (UTR). The genomic RNA encodes a single polyprotein of approximately 3000 amino acid that is cleaved into three structural and seven nonstructural proteins by cellular and viral proteases. The three structural proteins are required for virion assembly and the nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) are all necessary for genome replication (Lindenbach and Rice, 2003).

In mammalian cells, a subset of cellular proteins involved in mRNA remodeling, decay and translational repression colocalize in discrete cytoplasmic domains referred to as processing (P), XRN1 or GW bodies (Eulalio et al., 2007; Eystathioy et al., 2002, 2003). The components of P-bodies include the decapping proteins DCP1a–DCP2 complex, the CCR4–CAF1–NOT deadenylase complex, and the 5′–3′ exonuclease XRN1 (Bagga et al., 2005; Behm-Ansmant et al., 2006; Giraldez et al., 2006; Wu et al., 2006). In addition the P-bodies contain a set of decapping activators such as the heteroheptomeric LSM1-7, proteins involved in the RNAi pathway such as the Ago proteins and GW182, as well as the DEAD-box helicases DDX3 and DDX6 (RCK/p54). P-bodies are thought to be sites where untranslated mRNAs are stored for later release or where mRNAs undergo decay and translational repression.

The genomic RNA of positive sense RNA viruses (like WNV) serves essentially as mRNA that is used for translation of viral proteins. However during viral replication, the RNA has to exit from translation to serve as templates for packaging into new virions. Thus not surprisingly, many RNA viruses interact with P-bodies in some way (Beckham and Parker, 2008). For example in Yeast, Brome mosaic virus genomic RNA and viral replication factors accumulate in P-bodies. In mammalian viruses, DDX3 is required for cytoplasmic transport of HIV genomic RNA (Garbelli et al., 2011; Yedavalli et al., 2004). DDX3 also interacts with hepatitis C virus core protein and DDX3 as well as DDX6 are required for HCV replication (Angus et al., 2010; Ariumi et al., 2007; Jangra et al., 2010). A recent study has reported that HCV hijacks many of the P-body and stress granule components into lipid droplets involved in HCV replication (Ariumi et al., 2011). Recently, we and others have reported that P-bodies are greatly diminished during West Nile virus infection (Chen et al., 2011; Emara and Brinton, 2007). However, what happens to P-body proteins during WNV infection remains to be determined. Thus, in this study, we examined the fate of P-body components during WNV infection.

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Results and discussion

Since microscopically visible P-bodies disappear after WNV infection, we first tested if the P-body proteins are depleted following infection. Western blot analysis was done on cell lysates of uninfected or WNV infected (MOI 2 and 36 h PI) HeLa cells. Interestingly we found similar protein expression levels of DDX3, DDX6, XRN1, LSM1, GW182 and DCP1a in uninfected and WNV infected HeLa cells (Fig. 1). For GW182, we have recently reported that its mRNA level indeed increases following WNV infection (Chen et al., 2011). We also measured the mRNA expression levels of DDX3, LSM1, Ago2 and Dicer in WNV infected cells and found that the mRNA levels were indeed increased by 2–4 folds (data not shown). The reason why mRNA levels are increased although protein levels remain the same is not clear. Protein half-life may be decreased following infection or alternatively, certain cellular miRNAs may repress protein expression following infection. More importantly, our results show that the P-body disruption is not a result of depletion of P-body components. This is in agreement with a recent study which showed that in HCV infected cells, although P-bodies are disrupted, the protein levels of P-body components were unaffected (PérezVilaró et al., 2012). This is also consistent with our previous finding that both siRNA and miRNA pathways are intact following infection (Chen et al., 2011).

Because P-body component proteins remained intact although P-bodies were disrupted, we investigated the possible interaction of WNV with the P-body proteins. Specifically, we examined the localization of GW182, LSM1, DDX3, DDX6, XRN1, XRN2, DCP1a and EDC4 proteins with respect to viral NS3 (which accumulates at viral replication sites) in infected HeLa cells using immunofluorescence and confocal microscopy. The cells were infected with WNV at MOI of 2 and 36 h post-infection the cells were fixed, permeabilized, blocked and stained for WNV using NS3 antibody. The cells were also stained with GW182, LSM1, DDX3, DDX6, XRN1, XRN2, DCP1a or EDC4 antibodies and the nuclei were stained with DAPI. We observed that in the uninfected HeLa cells, the P-body proteins localized in discrete cytoplasmic P-bodies and/or dispersed in the cell cytoplasm to varying extents. In striking contrast, in infected cells, GW182, LSM1, DDX3, DDX6 and XRN1 were intensely concentrated in perinuclear regions and colocalized with the viral NS 3 (Fig. 2a–e). However, the P-body proteins, DCP1a and EDC4 or the non P-body XRN-2 did not colocalize with NS-3, but were diffusely distributed in the cytoplasm or nucleus (XRN 2) (Fig. 2f–h). These results suggest that many, but not all components of P-bodies are recruited to viral replication sites. Although DCP1a and EDC4 were not recruited to viral replication sites, they were not visualized in P-bodies after infection (but rather distributed in the cytoplasm). This is probably because in the absence of essential components like GW182, LSM1, DDX3 and XRN1, the P-bodies could no longer be detected as discrete microscopic foci. Why DCP1a and EDC4 are not recruited to WNV replication sites, although other P-body proteins are, is not clear. However similar to our results, Scheller et al. (2009) found that DCP1a was not required for translation and replication of HCV virus, whereas other P-body components like LSm1-7 were needed. XRN2 is a nuclear protein and does not concentrate in P-bodies and we used it as an unrelated protein marker to show the specificity of redistribution of P-body components.

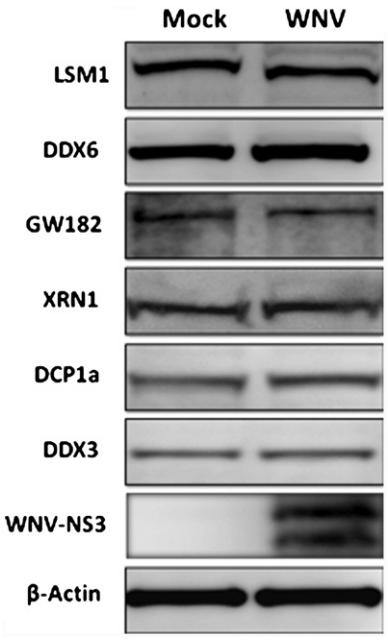

Fig. 1. WNV infection does not alter the protein levels of P-body components. Uninfected (mock) and WNV infected (36 h post-infection) HeLa cell lysates were analyzed for the expression of indicated proteins by Western blot using antibodies to LSM1, DDX6, GW182, XRN1, DCP1a, DDX3, and WNV NS3. β-actin served as loading control. The experiment was repeated twice with similar results.

We also confirmed that viral replication intermediates are concentrated in the perinuclear regions using an antibody against double-stranded RNA (Fig. 2i). Moreover, upon staining the infected cells with viral NS3 or dsRNA antibodies along with the endoplasmic reticulum (ER) marker, calnexin, we observed that both dsRNA and NS3 colocalize with calnexin (Fig. 2j). This is consistent with the fact that viral RNA replication occurs inside perinuclear replication complexes located inside vesicles that are invaginations of rough ER membranes (Gillespie et al., 2010). The newly transcribed genomic viral RNA exits the replication complex through the neck of these vesicles and is either used for translation of viral proteins or binds to capsid proteins associated with ER membranes leading to virion budding into the lumen of the ER (Gillespie et al., 2010). Further studies are necessary to determine the exact role of P-body components in these processes.

Next, we studied the time course of disruption of P-bodies, relocalization and the colocalization of LSM1, GW182, DDX3 and XRN1 with WNV NS3 at the viral replication sites. We infected HeLa cells with WNV at a MOI of 1. Cells were fixed 12, 24 and 36 h post-infection, permeabilized, blocked and immunostained for P-body components as described earlier. In uninfected cells, GW182, LSM1, XRN1 and DDX3 were concentrated in punctate P-bodies dispersed throughout the cell cytoplasm. In response to WNV infection at 12 h PI, we observed a decrease in both the number and brightness of P-bodies and an increase in the presence of LSM1, GW182, DDX3 and XRN1 in the cell cytoplasm. At 24 h PI all these proteins started accumulating around the nucleus at the virus replication site, and their colocalization with WNV NS3 was evident. At 36 h PI, the proteins were completely recruited and very little cytoplasmic staining was visible (Fig. 3a–d). These results suggest that early after infection, P-bodies are disrupted and the component proteins released into the cytoplasm. Active recruitment at viral replication sites starts at 24 h and is rapidly completed by 36 h PI.

P-body components can differentially affect virus replication. For e.g. RCK/p54, GW182, LSM1 and XRN1 suppress HIV infection and silencing these proteins enhances viral replication (Chable-Bessia et al., 2009; Nathans et al., 2009). On the other hand, many P-body components are needed for efficient replication of Hepatitis C virus (Ariumi et al., 2007, 2011). Therefore, we asked whether P-body components are negative or positive regulators of WNV replication. We transiently transfected HeLa cells with smart pool siRNA for each of LSM1, DDX3, Ago2, Dicer and GW182 proteins and a non

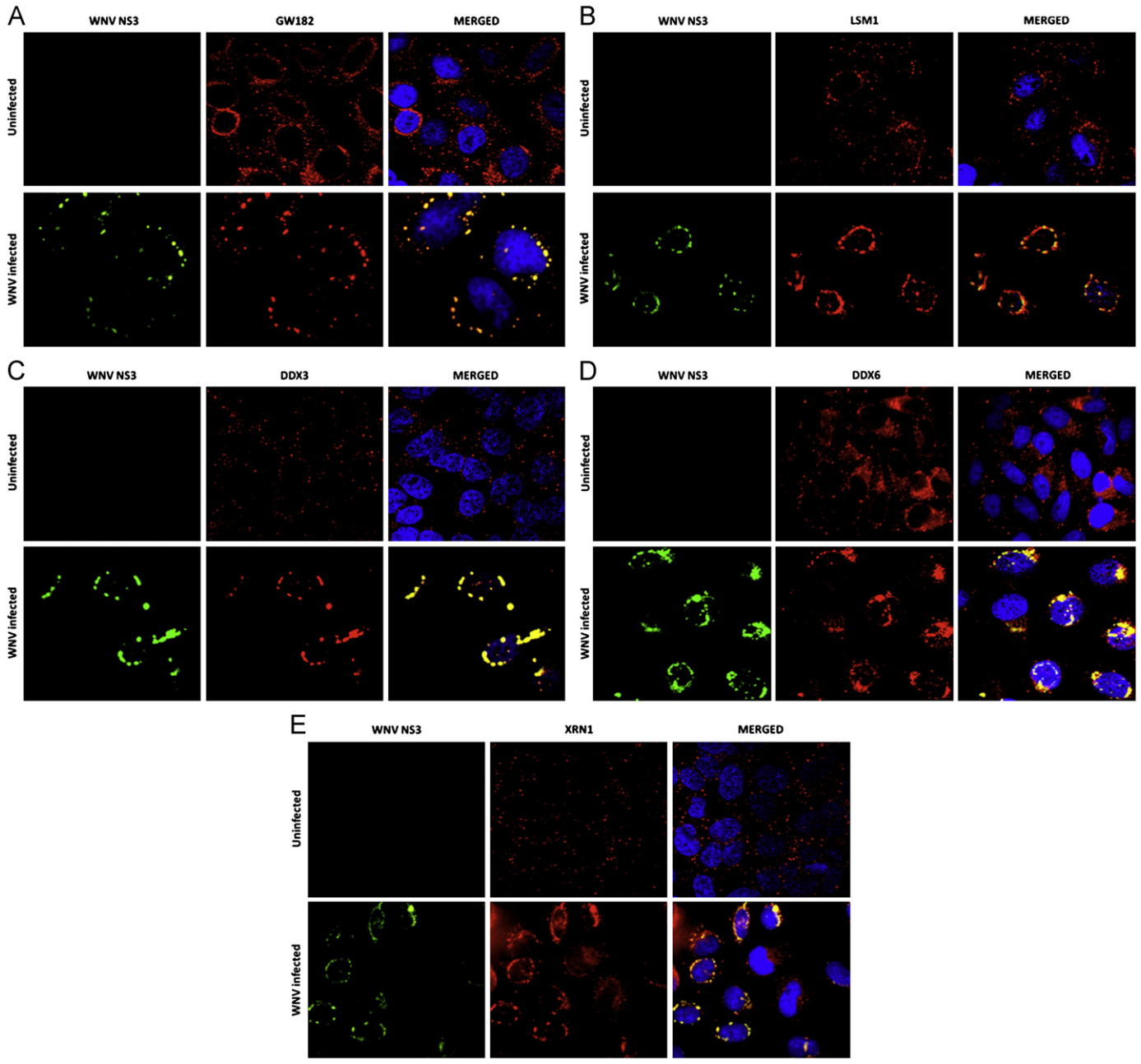

Fig. 2. Several P-body components are recruited to WNV replication sites. Uninfected and WNV infected (36 h post-infection) HeLa cells were stained with DAPI, WNV NS 3 antibody (detected using FITC labeled secondary antibody) and GW182 (a), LSM-1 (b), DDX3 (c), DDX6 (d), XRN1 (e), DCP1a (f), EDC4 (g) XRN2 (h), dsRNA (i) antibodies (detected using Alexaflour^{594} labeled secondary antibody) and examined by confocal microscopy. NS3/dsRNA costaining with ER marker calnexin is shown in (j). Individual antibody stained as well as merged images are shown as indicated. Each experiment was repeated 2–3 times.

P-body cellular protein, GAPDH as control. These siRNAs effectively knocked down the corresponding proteins both at mRNA and protein levels as determined by qRT-PCR and Western blot respectively (Fig. 4a and b). To determine if knockdown of P-body components affect viral replication, the siRNA treated cells were infected with WNV and viral replication monitored by FACS analysis and qRT-PCR at 36 h PI. In LSM1 siRNA treated cells, there was a ~70% reduction in viral replication as indicated by reduction in infected cells revealed by FACS analysis and viral RNA levels revealed by qRT-PCR. For other P-body components, we observed a moderate decrease in WNV infection levels (~30%–50%) with the least decrease observed in Dicer silenced cells (Fig. 4c and d). However, knockdown of a non-P-body protein GAPDH did not influence WNV replication (Fig. 4e), demonstrating the specificity of the effect of P-body component knockdown. These results suggest that the P-body components are required for efficient WNV replication.

Taken together, our results show that many P-body components are recruited to WNV replication sites and positively regulate viral replication. More work is needed to determine how exactly the P-body components influence WNV replication. The 5′ end of the genome of WNV contains a type I cap but the 3′ end lacks a poly-(A) tail. Although the 5′ cap of WNV enables recruitment of the 40S ribosomal subunit via the eIF4F cap-binding complex, because of lack of ploy-(A) tail, WNV RNA cannot assume a "closed loop" conformation (which enhances translation efficiency) due to lack of binding of the poly-(A) binding protein (PABP) or the PABP-interacting protein-1.

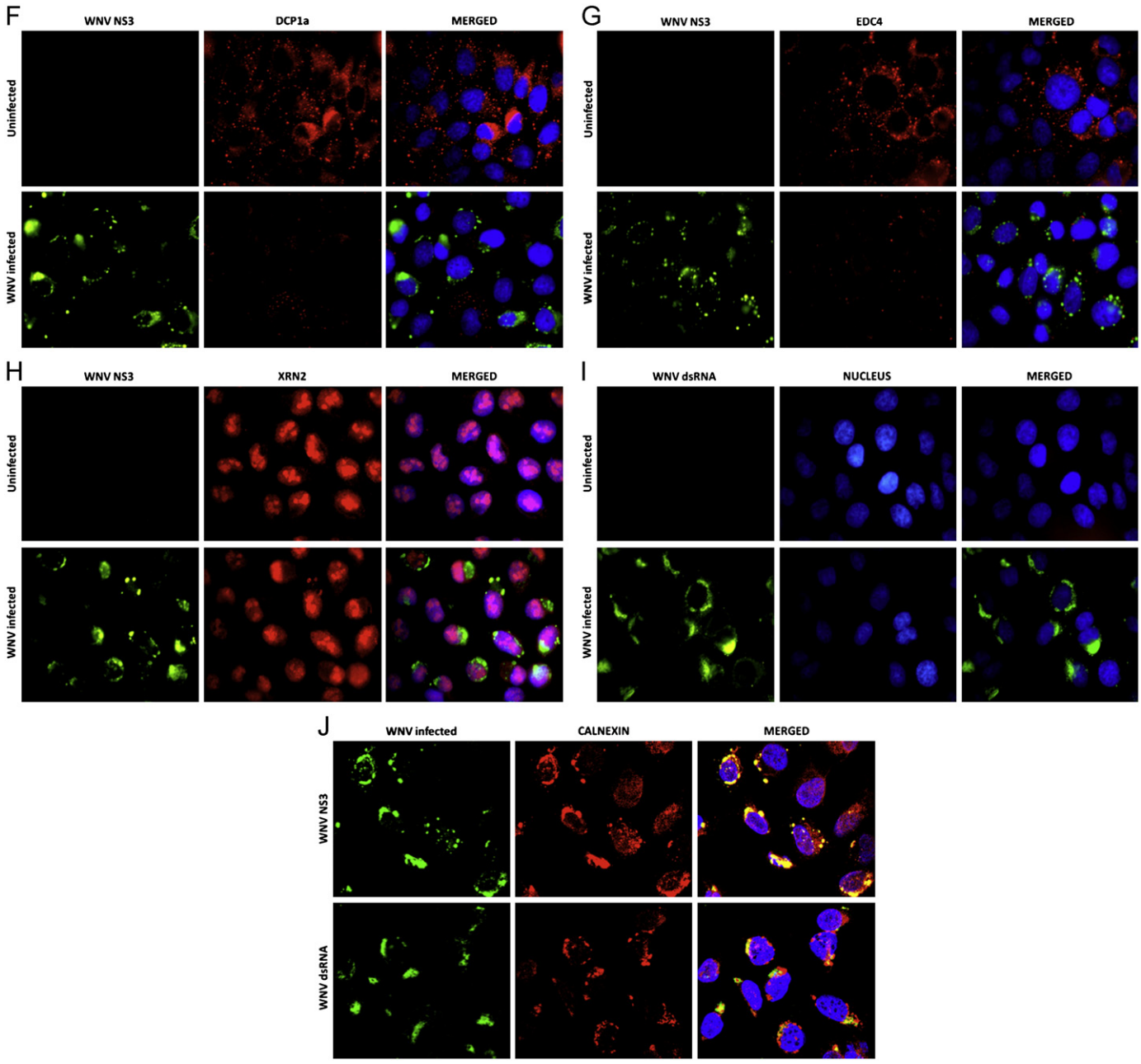

Fig. 2. Continued.

Interactions between the cellular proteins binding to the 3′ and 5′ terminal genomic RNA structure have been hypothesized to bridge the 5′ and 3′ termini (Brinton, 2002). In fact, several SG and P-body proteins including DDX6, G3BP1, G3BP2, Caprin1, and USP10, have recently been shown to bind DENV 3′ UTRs and that DDX6 assembly on the 3′ UTR is required for DENV replication (Ward et al., 2011). Thus, it is possible that P-body components might serve to bridge the termini of WNV RNA to enhance translation and/or replication. DDX6, LSM1, XRN1, PATL1 and Ago2 have also been shown to be recruited to HCV replication sites and it has been speculated that these P-body components might be recruited to aid the enhancement of HCV replication by the liver-specific miR-122. However, unlike HCV, no specific miRNA has been reported to regulate WNV replication and WNV shows no liver tropism. Thus, the recruitment of P-body components may not be particularly relevant in the context of miR-122, but is a common feature of flaviviral infection. This is also in line with a recent study that showed that DDX6 binds at specific sites in the dengue viral 3′ UTR and is required for efficient viral replication (Ward et al., 2011).

In addition to P-body components, several stress granule (SG) components including TIA-1/TIAR in WNV infection and G3BP-1 and PABP-1 in HCV infection are also recruited to viral replication sites. Moreover, the SG proteins G3BP1, G3BP2, Caprin1 and USP10 have also been shown to bind to different regions in the dengue virus 3′ UTR. The reason why so many components of P-bodies and SG are recruited to flaviviral replication sites remains to be determined, although it has been suggested that they might influence switching between translation and replication of positive sense viral RNA and/or viral assembly, packaging and egress (Beckham and Parker, 2008). It is noteworthy that different viruses use the P-body and SG components in different ways. For e.g., GW182, LSM1 and XRN1 appears to suppress HIV infection from within the P-bodies, whereas flaviviruses disrupt

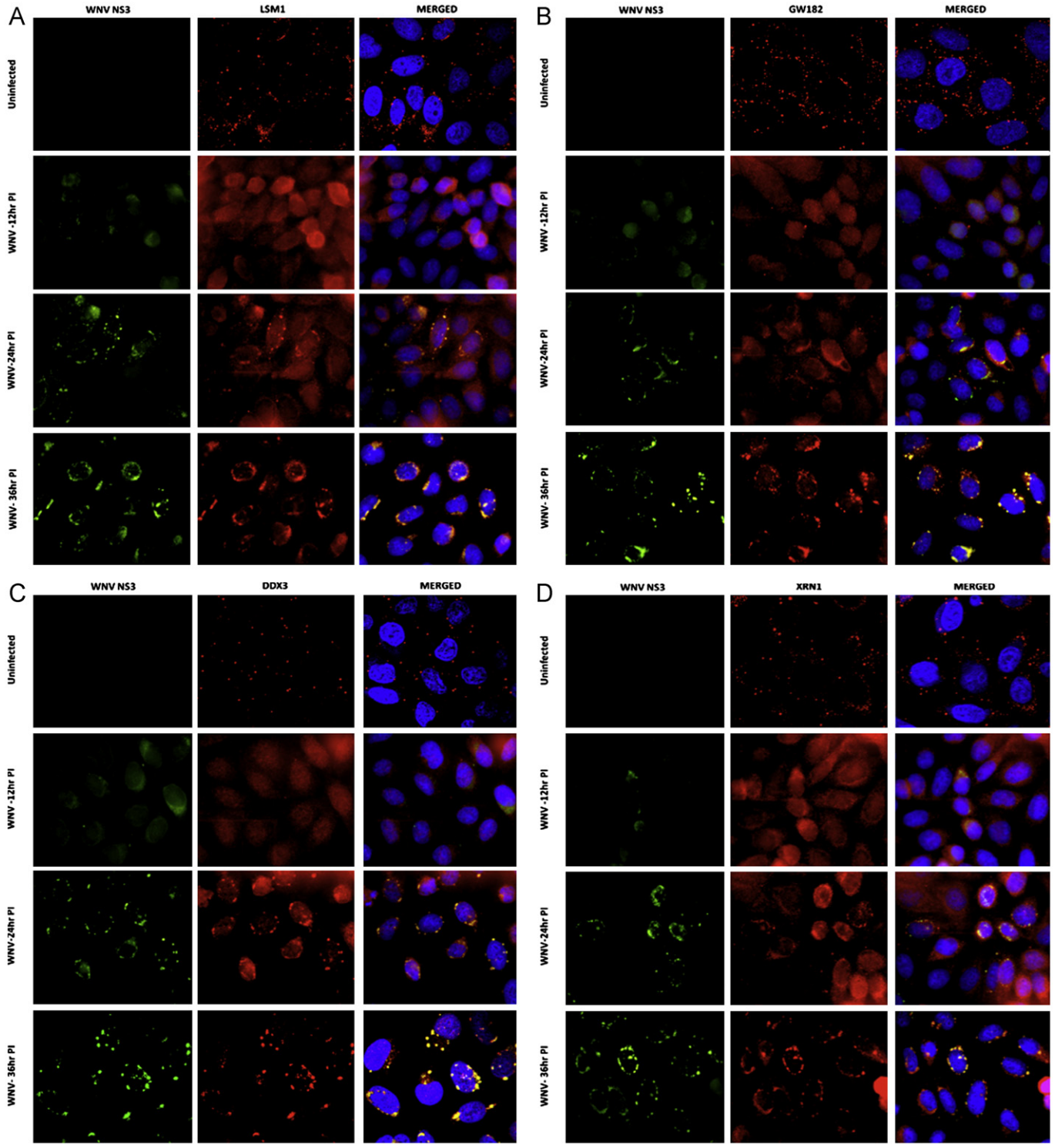

Fig. 3. Recruitment of P-body components to viral replication sites occurs late during WNV infection. Uninfected and WNV infected HeLa cells were analyzed for recruitment of LSM1 (a), GW182 (b), DDX3 (c) and XRN1 (d) at 12, 24 and 36 h post-infection as described in Fig. 2.

P-bodies and recruit components to enhance replication; HIV and poliovirus proteases cleave the PABP-1 and G3BP-1 to suppress SG formation (Dougherty et al., 2011), whereas SG formation is suppressed in HCV and WNV infection because of recruitment of SG proteins at replication sites. Thus, it appears that P-body and SG components play an important role in many virus infections and viruses hijack these host proteins to their advantage in different ways.

Materials and methods

Cell and virus culture

HeLa and BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). West Nile Virus (B956 strain, ATCC) was grown and plaque titered on BHK-21 monolayers.

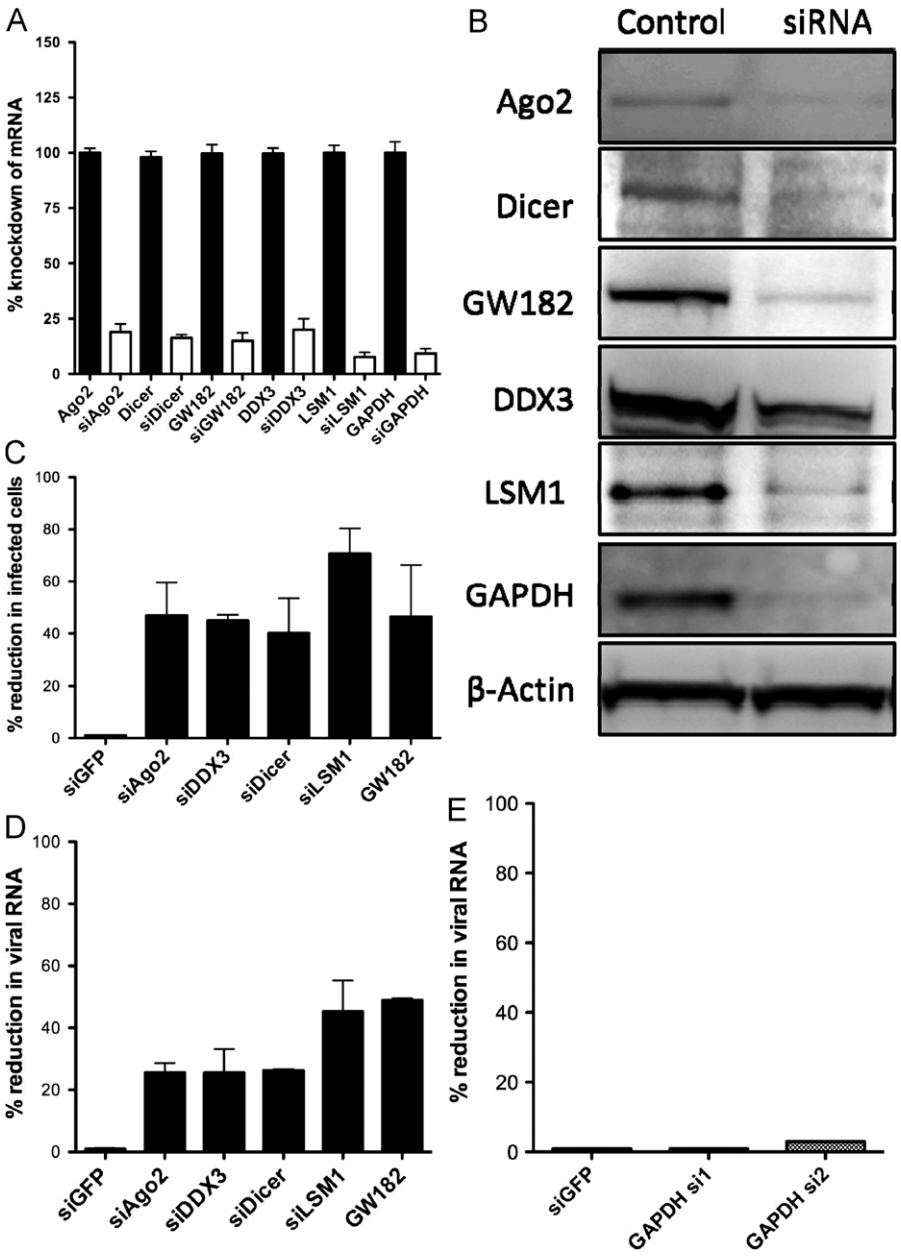

Fig. 4. P-body components positively regulate WNV replication. The siRNA knockdown of P-body components and GAPDH (an unrelated cellular protein control) was confirmed at both mRNA and protein level by qRT-PCR (a) and Western blot (b) respectively. mRNA (a) and protein (b) levels in untreated and corresponding siRNA treated cells are shown. β actin served as loading control for Western blot analysis in (b). To study the effect of P-body component silencing on WNV replication, HeLa cells were transfected with a pool of control siRNA (siGFP) or siRNAs specific for Ago2, DDX3, Dicer, LSM1 and GW182 siRNA using Lipofectamine 2000 and infected with WNV 24 h after transfection. Thirty-six hours post-infection, the cells were tested for virus replication by FACS analysis after staining with a WNV envelope specific antibody (c) and by qRT-PCR to detect WNV RNA levels (d). GAPDH silencing, used as an unrelated cellular protein control had no effect on WNV replication (e). Mean+SD of triplicate experiments is shown.

Western blot

HeLa cells were uninfected or infected with WNV at multiplicity of infection (MOI) of 2 and 36 h later, the cells lysed in a buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. Following a 30-min incubation on ice, lysates were clarified by centrifugation and protein concentrations were determined using a detergent-compatible protein assay kit (Pierce). Equal amounts of protein from these lysates were electrophoretically separated on 4%–12% Nu-PAGE gels (Invitrogen) by SDS-polyacrylamide gel electrophoresis, and electroblotted onto polyvinylidene difluoride membranes (Immobilon-P transfer membrane; Millipore). Following a blocking step with Tris-buffered saline containing 0.1% Tween-20 and 5% dry milk, the membranes were incubated with antibodies to LSM1, DDX6, GW182, XRN1, DCP1a, DDX3, Dicer, Ago2, WNV NS3 and GAPDH followed by horseradish peroxidase-conjugated secondary antibody (Peirce). Bound horseradish peroxidase was visualized with an ECL substrate kit (Thermo Scientific). Membranes were stripped and reprobed with anti β-actin antibody (4967; Cell Signaling) as a loading control.

Immunostaining

HeLa cells grown on cover slips were either uninfected or infected with WNV at indicated mois. At various times postinfection, the cells

were washed in PBS, fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked (2% bovine serum albumin, 5% normal horse serum, and 10 mM glycine in phosphatebuffered saline) at room temperature. The cells were then incubated with anti-GW182 (G5922; Sigma Aldrich) or anti-GW182 index human serum (Kindly provided by Dr. Marvin J. Fritzler), anti-LSM1 (LS-C97364; Life Span), anti-DDX3 antibody (54258 [IN]; Anaspec), anti-DDX6 (A300-460A; Bethyl), anti-XRN1 (A300-443A; Bethyl), anti-XRN2 (A301-103A; Bethyl), anti-DCP1a (AB47811; Abcam), anti-EDC4 (AB72408; Abcam), anti-dsRNA antibody J2 (J2-1101; English & Scientific) anti-WNV-NS3/2b (MAB29071; R&D systems) and anti-Calnexin (♯2433; Cell Signaling) antibody at different dilutions in PBS containing 3% bovine serum albumin (BSA) for 1 h at room temperature. The cells were then stained with appropriate secondary antibodies (AlexaFluor 594-conjugated anti-rabbit antibody and fluorescein isothiocyanate-conjugated anti-mouse antibody (Invitrogen)). Following extensive washing in PBS, the cells were mounted onto slides using a mounting medium ProLong Gold (P36935; Invitrogen) containing DAPI (4,6-diamidino-2-phenylindole) to stain nuclei and reduce fading. The cells were visualized for P-body components and WNV NS3/2b immunofluorescence staining using a Nikon Eclipse confocal/fluorescence microscope (Nikon USA).

RNA interference

The following small interfering RNAs (siRNAs) were used: human Ago2 (siGENOME SMRT pool M-013754-01-005), human DDX3 (siGENOME SMRT pool M-013754-01-005), human Dicer (siGENOME SMRT pool M-013754-01-005), human LSM1 (siGENOME SMRT pool M-005124-01-005), human GW182 (siGENOME SMRT pool M-005124-01-005), (Dharmacon, Thermo Fisher Scientific, Waltham, MA), human GAPDH (Sigma Aldrich) and GFP siRNA as a control. HeLa cells (1×10^5) were seeded in 24 well plates overnight and transfected in triplicate with the above mentioned siRNAs (100 pmole final concentrations) using Lipofectamine-2000 (Invitrogen) according to the manufacturer's instructions. The level of WNV infection was determined by FACS analysis and qRT-PCR. For FACS analysis, 36 h post-infection cells were harvested and stained with anti-flavivirus envelope specific antibody (4G2, ATCC). Total RNA isolated from the infected cells using RNeasy minikit (Qiagen, Hilden, Germany) was used for analysis of intracellular WNV RNA level by qRT-PCR. qRT-PCR was performed using a SYBR green PCR master Mix (Applied Biosystems) on 7900HT real-time PCR system. Amplification conditions were as follows: 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The 18srRNA was used as an internal control in all qRT-PCR assays and relative WNV viral RNA levels were normalized with 18srRNA mRNA and calculated using the δCt method. The forward and reverse primers sequences for 18srRNA were GAGAAGACGGTCGAACTTGACT and ACCTACGGAAACCTTGTTACGA respectively. The forward and reverse primers for WNV were ATCGCCGGACTTATGTTCG and CTTTCGCTAGAGCCTGTGATTT respectively.

Acknowledgments

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